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14. ABSTRACT Breast cancer metastases are usually found at the ends (metaphyses) of long bones where they cause osteolysis. The objective was to determine the trafficking of cancer cells in the marrow cavity and to identify factors that attract them. Human breast cancer cells that express green fluorescent protein (MDA-MB 231 ^{GFP}) were inoculated intracardially into athymic mice.; femurs harvested from 1 hr to 6 wk later and analyzed by fluorescence microscopy, immunohistochemistry, histomorphometry, flow cytometry and PCR. Single cells were detected within 1 hr in the distal metaphyses. Most cleared the marrow by 72 hr; but at 1 wk small foci formed in the ends near osteoblasts. At 2 wk the foci grew and coalesced. By 4 wk, the tumor masses were large and extended into the diaphysis. The osteoblasts were dramatically reduced (8% of control), while osteoclasts were reduced modestly (~60% of control). Ours is the first <i>in vivo</i> evidence that tumor cells influence not only osteoclasts, as widely believed, but also eliminate functional osteoblasts, thereby restructuring the bone microenvironment to strongly favor osteolysis. Using an ELISA array we also found that the metaphyseal bone was rich in several cytokines and factors that were only weakly detected in the shaft of the bone. Strategies that restore osteoblast function, perhaps by modifying the bone microenvironment, are needed to improve treatment of osteolytic bone metastases.					
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INTRODUCTION

Breast cancer cells frequently metastasize to bone where they cause osteolytic lesions. The bone loss is associated with fractures, bone pain and hypercalcemia and is a major medical problem (Body 1992, Coleman 1997). At the time of death, metastatic bone disease accounts for the bulk of the tumor burden (Mundy 2002). The tumor masses and the osteolytic lesions are usually found at the ends of long bones, i.e. the metaphyses. However, the basic biology of this event is not understood (Mundy 2002). For example, it is not known how breast cancer cells arrive at their destination and what attracts them to this site. The objective of this study was to determine the trafficking patterns of breast cancer cells early upon their arrival in the bone marrow cavity and to identify factors that attract the cells to the metaphyseal ends of the bone. The development of green fluorescent protein (GFP)-expressing breast cancer cells allowed us to detect metastases prior to bone loss (Harms et al. 2002). Athymic mice were inoculated with GFP-human metastatic breast cancer cells, MDA-MB-435 (MDA435^{GFP}) by intracardiac injection and the femurs harvested at various times from 1 hr to 6 wk. Some femurs were fixed and decalcified for immunohistochemistry and histomorphometry in order to detect the presence of the cancer cells and the state of the osteoblasts and osteoclasts. From other femurs, the marrow was collected from the distal, proximal metaphyses and from the shaft (diaphysis) of the bone marrow cavity. The cells from the marrow cavity were analyzed by flow cytometry for GFP positive cells and with quantitative real-time PCR for a human gene, HERVK. In addition marrow and bone from the metaphyses and diaphyses were incubated for 24 hr. The culture preparations were screened for the presence of cytokines and growth factors (Muller et al. 2001).

BODY

Task 1: To determine how metastatic breast cancer cells partition among the metaphyseal, endosteal, and cortical bone marrows.

The first task was to determine how metastatic breast cancer cells partition between the metaphysis and diaphysis; and, in the diaphysis, if they tended to be next to the bone (endosteal) or in the more central (cortical) bone marrow in the femurs from mice inoculated with MDA435^{GFP} breast cancer cells. We extended the study to examine separately the proximal (hip) metaphyseal region versus the distal (knee) metaphyseal regions of the femur.

We have carried out several experiments, some with a collaborator, Dr. Danny Welch, University of Alabama-Birmingham. A manuscript is in preparation (see Pushkar et al. under Reportable Outcomes). In the first experiment, athymic mice were injected by intracardiac injection (approximately 3×10^5 breast cancer cells). At various times, (1 hr, 2 hr, 4 hr, 8 hr, 24 hr, 72 hr, 1 week, 2 weeks, 4 weeks, 6 weeks) the femurs were harvested. Upon removal from the animal, the intact femurs were inspected with a fluorescent stereomicroscope. Tumor cells could be seen in the bone (Figure 1). They were first noted in the distal ends of the femur (Figure 1A). *(Please note that figures are found below)*. Numerous solitary cells were seen after 1 hr of injection, (Figure 1B), and fluorescent foci were seen by 1 week. These coalesced into larger foci (Figure 1B, A2-A3) and tumor masses, sometimes filling the whole marrow cavity (4-6 weeks Figure 1B, A4 and Figure 2 G). Early after inoculation, tumor cells were more readily seen in the metaphysis before they could be seen in the diaphysis (Figure 1A).

Sections of fixed and decalcified bone were examined by anti-GFP immunohistochemistry (Figure 2A) or examined directly by fluorescence confocal microscopy (Figure 2 E). We detected approximately the same numbers of cells using both techniques. It was difficult to find single cells in individual sections of femurs taken from mice before about 1 wk post inoculation (note individual cell in Figure 2 B1). However, at later times the MDA-435^{GFP} cells were easily detected (Figure 2 B2-4, E1-3). Masses of tumor cells were seen in the marrow cavity by 4-6 weeks (Figure 2 F, G). A comparison of trabecular bone in femurs with a cancer mass (Figure 2D) was significantly less than that seen in bones from normal animals (Figure 2C) indicating osteolytic degradation.

Both flow cytometry and real time PCR (RT-PCR) were used to quantitate the numbers of cancer cells in the femur (Figure 3). We estimated that only a small fraction (0.01%) of the injected cells could be found in a femur (Table 1). These procedures also indicated the pattern of distribution of the cancer cells was similar to that seen with the histological approaches. It should be pointed out that localization is not as precise with these techniques because cutting the metaphysis from the diaphysis was not consistent. We also found that the tumor cells in the diaphysis were largely (>95%) near the endosteal bone and not in the central marrow (Figure 6).

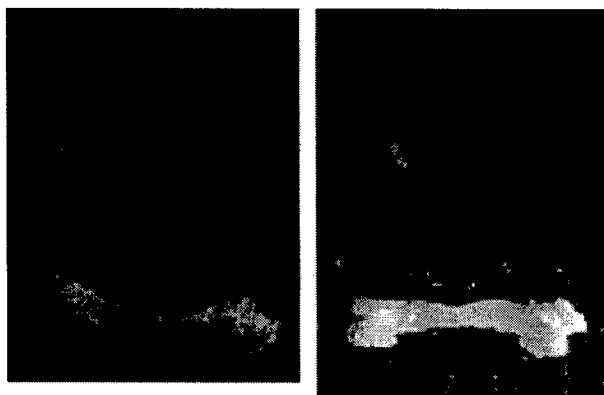
This pattern is similar to that seen with the movement of hematopoietic cells (Mason et al. 1989).

We had previously found with *in vitro* co-culture that breast cancer cells caused an increase in osteoblast apoptosis and that the osteoblasts did not differentiate into bone matrix forming cells (Mastro et al. 2004; Mercer et al. 2004). Therefore, we carried out histochemical analyses to determine if the cancer cells affected the osteoblasts the same way *in vivo*. Using a fluorescent TUNEL assay, we saw that apoptotic osteoblasts increased in femurs following the inoculation of the cancer cells (Figure 4D,G; Figure 5). We also noted that apoptotic osteoblasts tended to be within 50 microns of a cancer cell (Figure 5). Staining of cryosections of femur for bone alkaline phosphatase activity to assess the level of the maturity of the osteoblasts, revealed that the trabecular bone from the cancer bearing animals had very little alkaline phosphatase activity compared to control bones (Figure 4H,I). The alkaline phosphatase activity in the growth plates of the bone was similar, however. These data together indicated that in the cancer bearing bone, the osteoblasts in the trabecular bone were undergoing apoptosis and also were unable to differentiate. Histomorphometry confirmed that there were significantly fewer osteoblasts in bone of cancer bearing mice (Figure 4B). A comparison of the ratios of calcified bone volume/tissue volume indicated that calcified bone was significantly lost by 4 weeks.

We also stained sections for tartrate-resistant acid phosphatase to detect osteoclasts. The numbers of osteoclasts remained relatively constant until later times when their numbers decreased (Figure 4E, F). This somewhat surprising loss of osteoclasts was confirmed by histomorphometry.

In summary, (Figure 6), the trafficking experiments indicated that early after their arrival in the bone, the cancer cells move to the metaphyseal regions. At least in the mouse, they tended to go to the distal metaphysis before they moved to the proximal. Most cancer cells were cleared from the bone by 24 hr and did not begin to proliferate until after 72 hr. The presence of the cancer cells led to loss of osteoid volume, to a decrease both in the numbers of osteoblasts and osteoclasts. The presence of the cancer cells led to a dramatic restructuring of the bone micro-environment.

Figure 1. A. Detection of MDA-MB 435^{GFP} cells in whole bone using fluorescent stereomicroscopy.



Femurs were removed from mice 4wk following inoculation with MB-435^{GFP} cells and visualized by bright field (bottom) and fluorescent microscopy (A). At higher magnification (B), fluorescent tumor cells were seen first in the distal ends of the bone. Single cells were detected by one hour (A1), and foci were apparent at 1 wk (A2). These foci became larger at 2 wk (A3). Tumor masses filled the marrow cavity by 4 wk (A4).

Figure 1B. The kinetics of MDA-435^{GFP} metastatic growth in the femur following intracardiac injection.

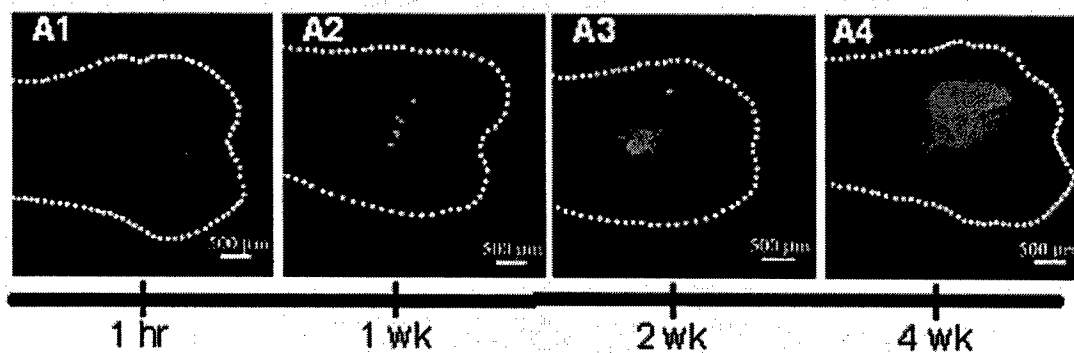


Figure 2. Detection and kinetics of MDA-435^{GFP} cells by immunohistochemistry and fluorescence microscopy in sections of femurs taken from mice at various times following inoculation. Panel B, images of sections fixed, decalcified and paraffin embedded sections stained with anti-GFP immunohistochemistry (brown) at various times after inoculation. B1 (1 hr) shows a single cell; B2 (1 wk) shows a small cluster. B3 (2 wk) and B4 (4 wk), tumor masses. Panel E illustrates detection of MDA-435^{GFP} cells by fluorescence microscopy. E1, 2wk; E2, 4wk; E3, 6 wk. E1', E2' and E3' are composites of phase and fluorescent images. Panel C and Panel D represent distal ends of femur stained with Goldner's trichrome stain (Panel C = normal bone; Panel D = 4wk). The amount of trabecular bone (teal) is significantly lower in bone containing tumor cells indicating osteolytic degradation. Representative Panel F (bright field) and Panel G (fluorescence) are images of a mouse femur at 4wk showing two large metastatic foci, one at each end of the femur. The distal end shows an iatrogenic fracture presumable due to weakness caused by tumor cell-induced osteolysis.

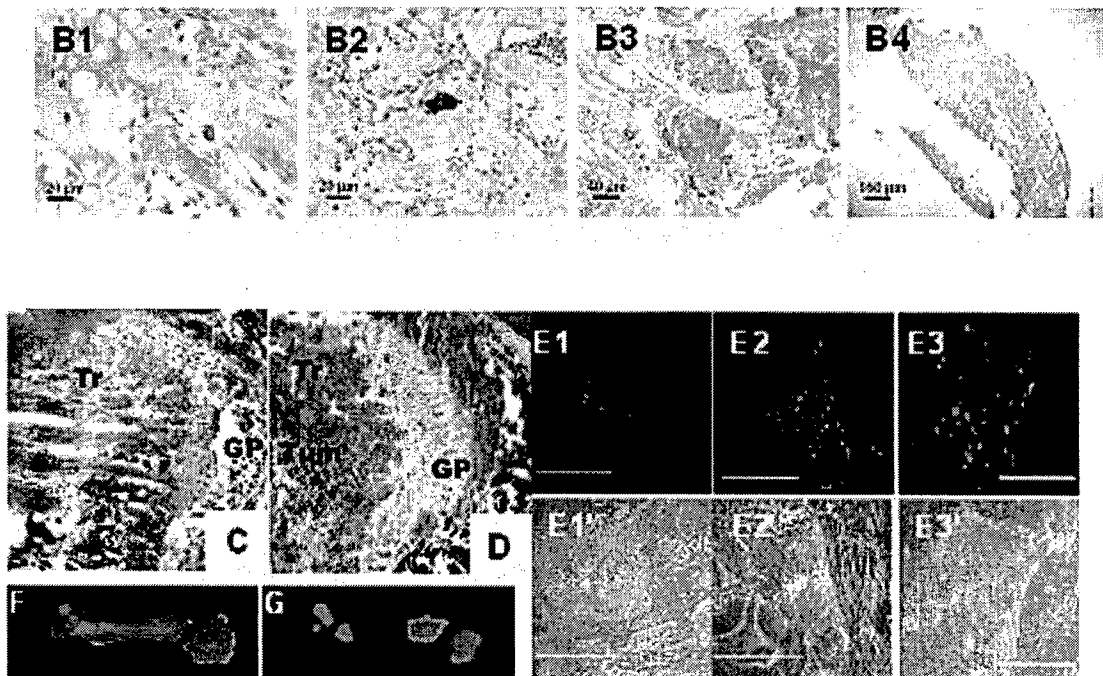
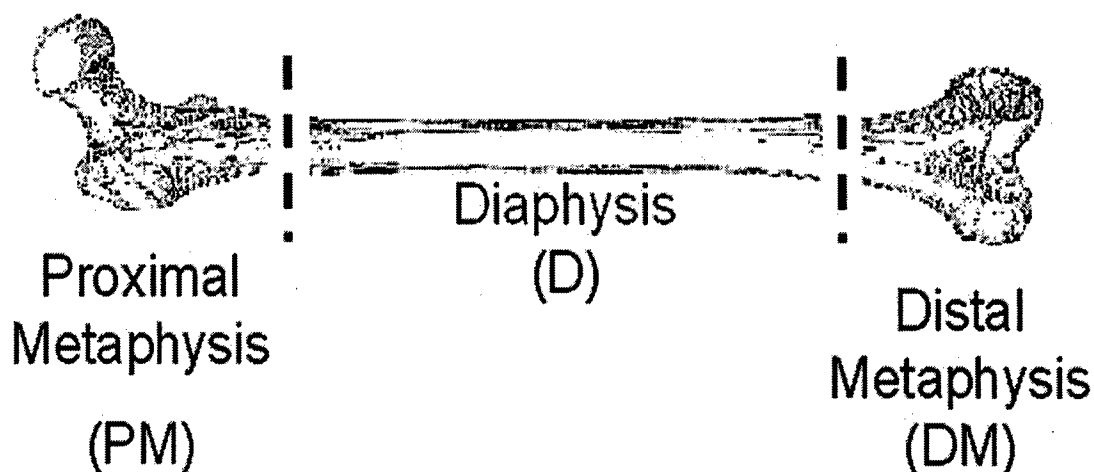


Figure 3. Detection of MDA-435^{GFP} metastatic cells in the metaphyseal and diaphyseal ends of the femur by flow cytometry or RT-PCR at various times following intracardiac inoculation.



Time after injection	Flow cytometry			Real-time PCR		
	PM	D	DM	PM	D	DM
1 hr	1/5	2/5	0/5	1/5	1/5	0/5
4hr	2/5	2/5	0/5	1/5	2/5	1/5
24 hr	0/5	0/5	0/5	0/5	0/5	0/5
72 hr	2/5	1/5	2/5	1/5	0/5	0/5
1 wk	1/5	1/5	0/5	1/5	3/5	2/5
4 wk	2/5	2/5	3/5	3/5	2/5	5/5
6wk	0/3	1/3	2/3	2/3	2/3	2/3

Femurs were removed and the ends removed from the shaft with a scissor. The proximal, and distal metaphyses and the cortical area were flushed with PBS and the cells collected and prepared for flow cytometry or DNA isolation and analysis as described in the methods section. The limit of detection of MDA-435GFP cells by flow cytometry was approximately 200 cells; by PCR it was about 150. Shown are the numbers of animals in the group in which the cancer cells were detected and the bone areas in which they were found.

Table 1. Retention of MDA-435^{GFP} cells in the femur following intracardiac injection

Time post-injection	Number of MDA-435 ^{GFP} cells in the femur	
	Mean	Range
1 hr	41	16; 104
4 hr	54	5; 529
24 hr	40	7; 270
72 hr	44	11; 180
1 wk	41	20; 82
4 wk	11,271	2,893; 43,915

Marrow containing tumor cells was flushed from femurs with a syringe. The numbers of MDA-435^{GFP} cells were determined by real-time, quantitative PCR using probes for the human gene, HERVK. Shown are log-transformed data for 5 mice per group.

Figure 4. MDA-435^{GFP} breast cancer cells diminished osteoblast (OB) and osteoclast (OC) numbers in colonized bone as evaluated by quantitative bone histomorphometry, immunohistochemistry and fluorescent microscopy. Panels A-C, histomorphometric analyses. (A, Bone volume to tissue volume; B, Number of OB per bone surface; C, Number of OC per bone surface). Panel D, the number of apoptotic OB (TUNEL positive) per linear bone surface at times following inoculation of tumor cells. Panel E, the number of OC (staining for tartrate resistant acid phosphatase, TRAP) per linear bone surface at times following inoculation of tumor cells. Panels A-E, * indicates significantly different (p.0.05) from normal bone. Panel F, representative image of OC staining for TRAP (red stain highlighted with white arrows) taken from a section of femur 2 wk after tumor cell inoculation. Bar = 25 μ m. Panel G, merged photomicrograph of MDA-435^{GFP} tumor cells (green) surrounding apoptotic OB (red by TUNEL using Cy-5 probe) taken from a femur 6 wk following inoculation. Bar = 25 μ m. Panels H, I, J, cryosections from a femur taken 4 wk after tumor inoculation. Bars = 100 μ m. H,I, stained for alkaline phosphatase activity (blue staining highlighted by red arrows) indicative of OB; J, merged fluorescent and phase images showing trabecular bone (TB) surrounded by cells (BC). Alkaline phosphatase activity was greatly diminished in the trabecular bone of tumor-bearing femurs but was still present in the growth plate (GP).**

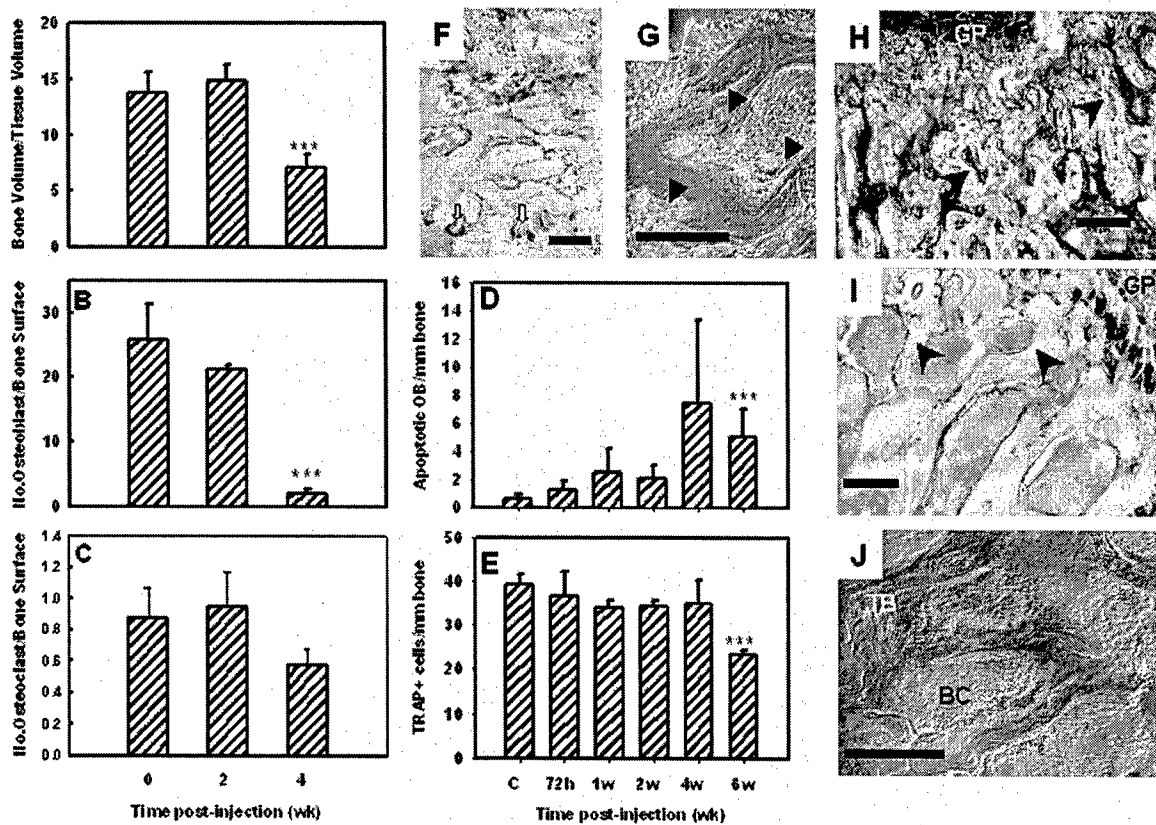


Figure 5. The presence of metastatic breast cancer cells in close proximity to apoptotic osteoblasts increased with time following inoculation of MDA-435^{GFP}. **Panel A**, apoptotic OB, detected by TUNEL, were counted in proximal and distal ends of paraffin sections of femur at times following tumor cell inoculation. **Panel B**, number of MDA-435^{GFP} cells within a 50 μ m radius of each apoptotic OB. Shown are the averages from three femurs per time. Proximal femur (hatched); distal femur (stippled); average over femur (solid). Apoptotic osteoblasts in the diaphyses were extremely rare.

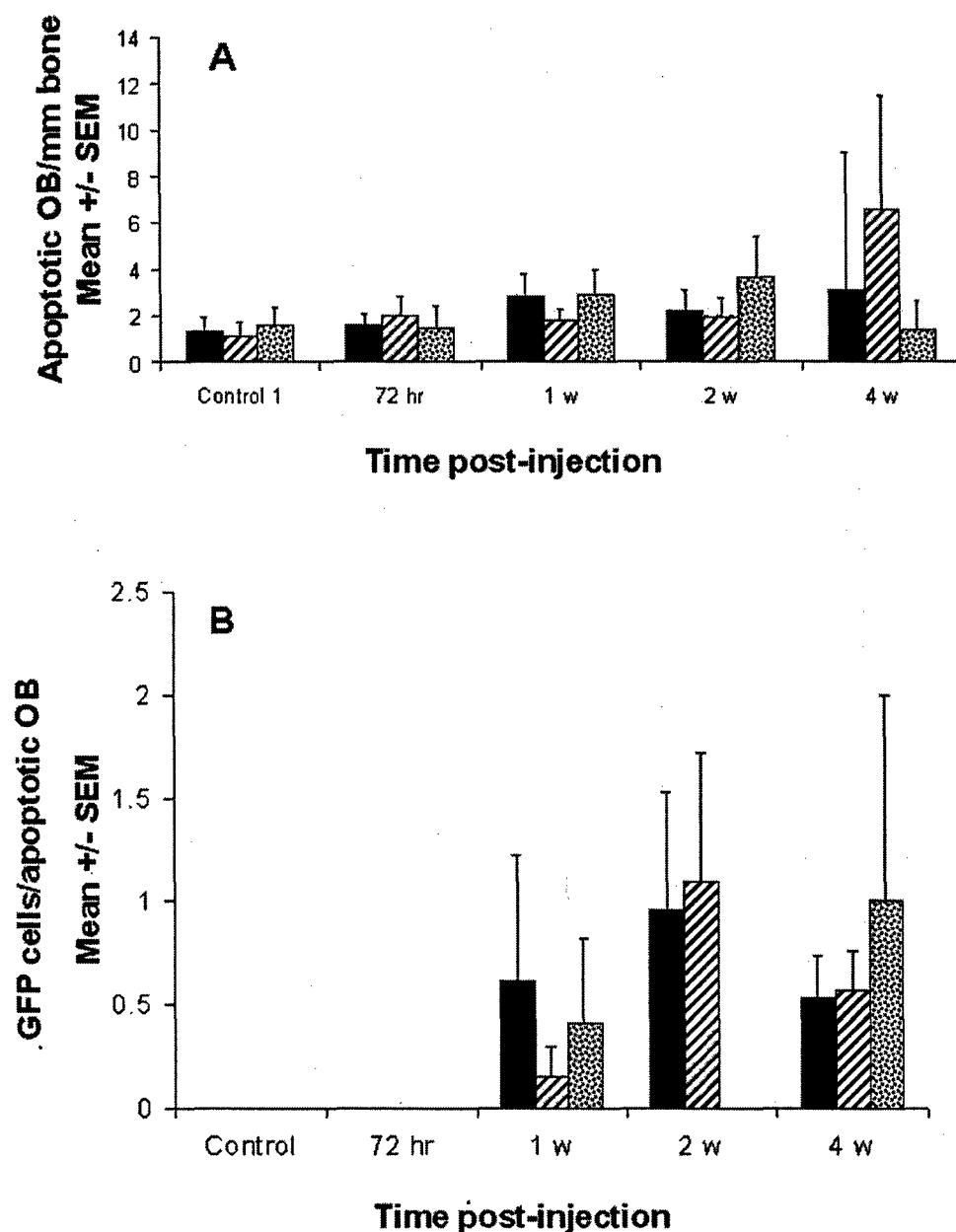
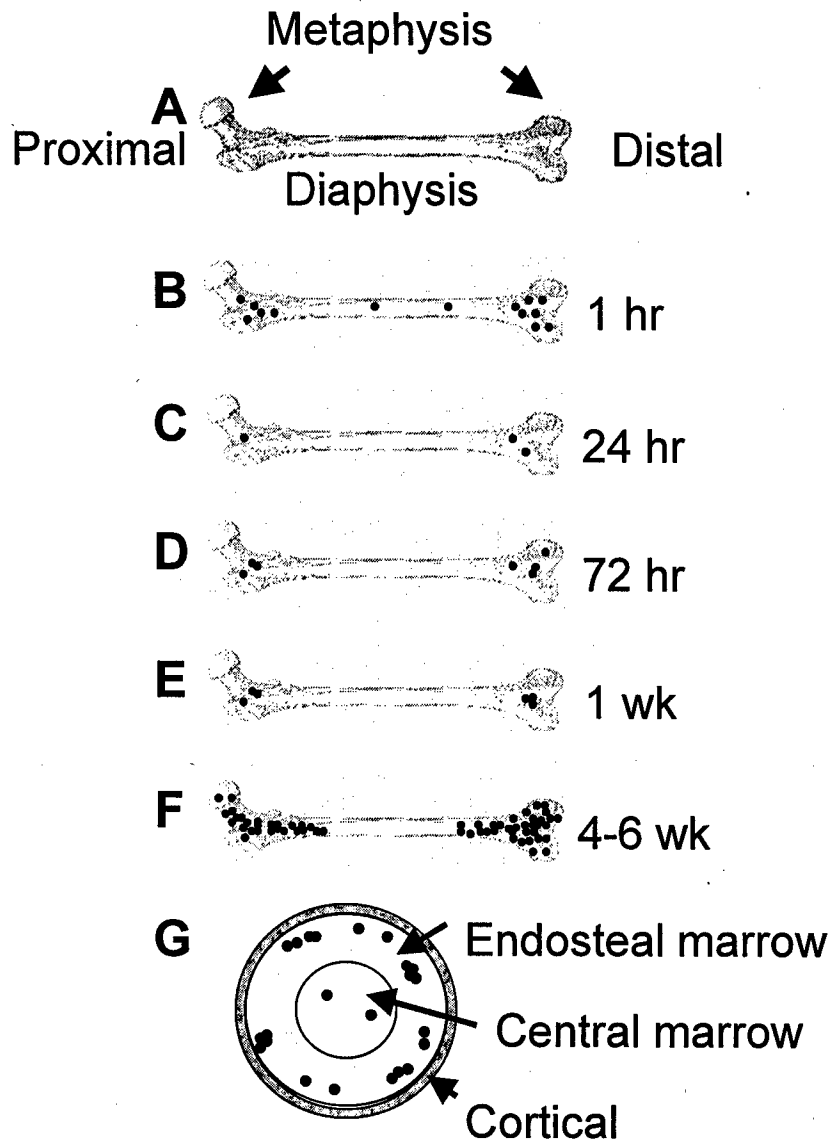


Figure 6..Schematic diagram depicting colonization of the femur by MDA-435^{GFP}. A normal femur is diagramed and labeled for reference (**Panel A**). Single cells (●) arrive in the bone marrow within 1 hr after intracardiac injection (**Panel B**), with a distribution proportionate to the relative blood flow to regions of the bone. Most cells arresting in the bone are cleared within 24hr (**Panel C**). Of those remaining, the majority are still single cells and are located in the metaphyses. A fraction of the surviving cells begin to proliferate after 72 hr (**Panel D**) with little change in the number of foci, or size of tumor cell clusters, at one week post-inoculation (**Panel E**). The lesions progressively grow so that by 4-6 wk, the mass of the metastases is large and the number of independently seeded cells indiscernible because the foci have coalesced. Despite not seeding and remaining in the diaphysis, metastases extend into the bone shaft as the lesions grow (**Panel F**). Flushing of bone marrow in established metastases revealed that most of the tumor cells are found in endosteal marrow (~90%) or in the central marrow (~10%), but never in the cortical bone of the diaphysis, as depicted in a cross-sectional view (**Panel G**).



Task 2. To compare chemotactic properties of metaphyseal bone and marrow with endosteal and cortical bone and marrow .

The second task was to compare the chemotactic properties of metaphyseal bone and marrow with endosteal and cortical bone and marrow. We proposed to prepare bone and marrow cultures; assay breast cancer cells for chemotaxis to the various bone and marrow preparations and assay culture medium from the bone marrow and bone preparations for cytokines and chemokines.

We isolated the femurs from non-cancer bearing mice and divided them into the metaphyses and diaphyses in order to screen for the presence of possible factors that would affect the metastatic cancer cells. The bone pieces and marrow were separated and cultured for 24 hr. The culture media were collected and screened for the presence of cytokines and factors using the RayBio Mouse Cytokine Antibody Array III & 3.1 which can detect 62 different mouse cytokines. Of the 62, we detected at least 15 in the metaphysis and of those 6 were also in the diaphysis (Table 2). Several inflammatory cytokines, IL-6, MIP-2, KC, MCP-1, were present as were adhesion molecules and chemokines. For some, e.g., IL-6, there appeared to be a gradient between the ends and the mid- section of the bone. We repeated this experiment except that we separated the marrow from the bone and incubated the marrow and the calcified bone separately. Almost all of the cytokines were found in the culture medium from the calcified bone and not in that from the marrow. Because of the expense we have not yet carried out ELISA to quantitate the cytokines.

We plan to carry out a similar experiment with femurs from animals after inoculation of the cancer cells to determine how the cancer cells modify the bone microenvironment. We plan to submit grants to obtain funds to carry out the numerous ELISAs and the chemotactic assays.

Table 2. Cytokines and Growth Factors Detected in the Metaphysis and Diaphysis of Mouse Femurs

Metaphysis	Diaphysis
IL-6	IL-6
L-selectin	L-selectin
P-selectin	P-selectin
Lymphotactin	Lymphotactin
VCAM-1	VCAM-1
GCSF	
KC	
MCP-1	
MIP-1 α	
MIP-2	
TIMP-1	
IGFPB-5	
sTNFR1 and R2	
INF γ	

Bolded cytokines appeared to be in higher amounts relative to the other cytokines.

KEY RESEARCH ACCOMPLISHMENTS

- Determined the pattern of appearance of metastatic breast cancer cells early upon arrival in femurs (1 hr to 6 weeks following inoculation).
- Determined that the cancer cells were found mainly in the metasphyses as opposed to the diaphysis from the earliest times. They also were detected in the distal metaphysis before the proximal metasphysis.
- Determined that tumor cells in the diaphysis were rare except late in the metastatic process. At that time the tumor cells were found in the marrow close to the endosteal bone.
- Quantitated the numbers of metastatic cells in the femur over time using flow cytometry and PCR.
- Determined that osteoblasts in femurs with cancer cell underwent apoptosis and did not differentiate.
- Determined the both osteoblasts and osteoclasts decreased in numbers as the tumor masses increased in size.
- Determined that the metasphyseal bone contains a variety of cytokines and adhesion molecules that were not detected in the diaphyseal bone.

REPORTABLE OUTCOMES

- A senior Biochemistry and Molecular Biology Major, also an Honors Student in the Schreyer Honors College, worked on part of this project for her honors thesis. She was the College of Science Student Marshall at graduation and is entering the graduate program in Molecular Biology of Cancer at Duke University in the fall. Her manuscript is in preparation.
- A junior Microbiology Major carried out part of this project for her undergraduate research requirement. She was accepted into a summer undergraduate program at the University of Pittsburgh based on her work. She will be a co-author on a manuscript submitted in August, 2005.
- A graduate student in Biochemistry and Molecular Biology worked on aspects of this

project. She graduated in Spring 2005 and is now a postdoctoral fellow at the University of N. Carolina. She is a co-author of the manuscript submitted in August.

- A first year graduate student participated in the RT-PCR aspects of this project and analyzed the data as part of an advanced statistics course. She is a co-author on the manuscript submitted in August.
- **Presentations (poster and platform) at the recent Era of Hope meeting.**

Mercer, R.J., P.A. Phadke, J.L. Jewell, C.V. Gay, V. Gilman, D.R. Welch, and **A.M. Mastro**. "Trafficking of breast cancer metastatic cells in bone." Era of Hope, Department of Defense, Breast Cancer Research Program Meeting, June 8-11, 2005, Philadelphia, PA.

Phadke, P.A., R. Mercer, J.F. Harms, J.C. Kappes, Y. Jia, A.R. Frost, **A.M. Mastro**, and D.R. Welch. "Kinetics of the early stages of breast cancer metastasis to bone." Era of Hope, Department of Defense, Breast Cancer Research Program Meeting, June 8-11, 2005, Philadelphia, PA.

- **Manuscript submitted August 2005.**

Pushkar A. Phadke*, **Robyn R. Mercer***, John F. Harms, Yijiang Jia, John C. Kappes, Andra R. Frost, **Jennifer L. Jewell**, **Karen M. Bussard**, **Sharkira Nelson**, Cynthia moore, **Carol V. Gay**, **Andrea M. Mastro**# and Danny R. Welch.# Kinetics of Metastatic Breast Cancer Cell Trafficking in Bone. Submitted, August 2005. (* equal first authors, # equal senior authors; Mastro, Penn State group is bolded).

CONCLUSIONS

Breast cancer metastatic cells follow a very specific route once in the marrow of the femurs. They either move or are cleared from the diaphysis and migrate to or grow in the metaphysis. In the mouse, they appeared in the distal end before the proximal end of the femur. Later, once tumor masses formed, the cells were found throughout the marrow cavity. One of the most

dramatic findings was the effect of the tumor cells on the osteoblasts. As predicted from our *in vitro* studies, the osteoblasts undergo apoptosis and are unable to function as bone repairing cells. We found in these present studies that *in vivo*, they diminished in numbers in the trabecular bone and that their alkaline phosphatase activity was also greatly reduced compared to bone from control mice. Somewhat unexpected was the finding that the osteoclasts also diminished in number as the tumor masses increased. Therefore the metastatic breast cancer cells led to a dramatic restructuring of the bone cells and their microenvironment such that osteolysis was strongly favored. The demise in osteoblasts helps to explain why treatment with bisphosphonates to inhibit osteoclasts is insufficient to promote bone healing. Comprehensive treatment needs to restore bone matrix as well as limit osteolysis.

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